

Estrogenic Potential of Certain Pyrethroid Compounds in the MCF-7 Human Breast Carcinoma Cell Line

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Estrogens, whether natural or synthetic, clearly influence reproductive development, senescence, and carcinogenesis. Pyrethroid insecticides are now the most widely used agents for indoor pest control, providing potential for human exposure. Using the MCF-7 human breast carcinoma cell line, we studied the estrogenic potential of several synthetic pyrethroid compounds *in vitro* using pS2 mRNA levels as the end point. We tested sumithrin, fenvalerate, *d-trans* allethrin, and permethrin. Nanomolar concentrations of either sumithrin or fenvalerate were sufficient to increase pS2 expression slightly above basal levels. At micromolar concentrations, these two pyrethroid compounds induced pS2 expression to levels comparable to those elicited by 10 nM 17 β -estradiol (fivefold). The estrogenic activity of sumithrin was abolished with co-treatment with an antiestrogen (ICI 164,384), whereas estrogenic activity of fenvalerate was not significantly diminished with antiestrogen co-treatment. In addition, both sumithrin and fenvalerate were able to induce cell proliferation of MCF-7 cells in a dose-response fashion. Neither permethrin nor *d-trans* allethrin affected pS2 expression. Permethrin had a noticeable effect on cell proliferation at 100 μ M, whereas *d-trans* allethrin slightly induced MCF-7 cell proliferation at 10 μ M, but was toxic at higher concentrations. Overall, our studies imply that each pyrethroid compound is unique in its ability to influence several cellular pathways. These findings suggest that pyrethroids should be considered to be hormone disruptors, and their potential to affect endocrine function in humans and wildlife should be investigated. **Key words:** 17 β -estradiol, MCF-7 human breast carcinoma cells, pS2, pyrethroid, RNA. *Environ Health Perspect* 107:173–177 (1999). [Online 21 January 1999] <http://ehpnet1.niehs.nih.gov/docs/1999/107p173-177go/abstract.html>

Reproductive dysfunction involving exposure to hormonally active compounds has been documented extensively in wildlife (1) and has been implicated in human breast cancer (2–4) and decreases in male-to-female birth ratio (5). As endocrine disruptors, many kinds of chemicals have been targeted for study in hormonally responsive bioassays.

Synthetic pyrethroids are analogs of a natural chemical moiety, pyrethrin, isolated from the chrysanthemum (6). The natural pyrethrin structure has been modified to be highly lipophilic and photostable, creating an effective pesticide and resulting in an increased presence in the environment (6). Human exposure to pyrethroid compounds is extensive. Worldwide, they are used as insecticides against ticks (7), mites (8), mosquitoes (found in bednetting as a control against malaria) (9,10), and as treatment for human head lice (11) and scabies (12).

Several studies on the effects of pyrethroids on thyroid hormone regulation and androgen function have been reported. Using rat models, Kaul et al. (13) showed fenvalerate to increase triiodothyronine (T₃) and thyroxine (T₄) levels in circulation inducing thyroid dysfunction (13). Aktar et al. (14) found that λ -cyhalothrin and bifenthrin reduced serum levels of T₃ and T₄ in the rat, whereas only λ -cyhalothrin could decrease the T₄/T₃ ratio (14). Among humans, a pyrethroid compound was

suggested to be the causal agent for gynecomastia in a group of Haitian men (15). Furthermore, several pyrethroid compounds appear to competitively inhibit testosterone binding to the androgen receptor and sex hormone binding globulin at high concentrations (16). Although limited, these reports suggest that some pyrethroid compounds are capable of disrupting endocrine function.

In a previous study using the Ishikawa Variant-I human endometrial carcinoma cell line, Garey and Wolff (17) showed that certain pyrethroid compounds have estrogenic potential. Since pyrethroid compounds have been shown to affect endocrine homeostasis, the purpose of our study was to confirm and further characterize the estrogenic potential of four synthetic pyrethroid compounds. Sumithrin, fenvalerate, *d-trans* allethrin, and permethrin, illustrated in Figure 1, were assayed for their ability to induce pS2 gene expression in MCF-7 cells. The MCF-7 human breast carcinoma cell line is a well-established *in vitro* system characterized by its estrogen responsiveness through expression of the estrogen receptor (ER) (18). The MCF-7 cells used in our studies express ER as determined by RNA polymerase chain reaction (data not shown). In these cells, expression of the pS2 gene product is directly induced by estrogen at the transcriptional level (19) and has been used as an end point for

xenoestrogens (20). Furthermore, cell proliferation of MCF-7 cells was also used to test the estrogenicity of chemical compounds.

Methods

Cells. MCF-7 cells [American Type Culture Collection (ATCC); Rockville, MD] were grown in DMEM (high glucose with L-glutamine) with phenol red, supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.1 mM nonessential amino acids, 4 mM L-glutamine, 0.1 mM sodium pyruvate, 10 μ g/ml insulin, and 10% fetal calf serum (Gibco BRL, Gaithersburg, MD). Cells were grown at 37°C with 5% CO₂ in a humidified incubator.

pS2 expression assay. MCF7 cells were plated at 30% confluency, supplemented with phenol red-free DMEM containing 5% charcoal dextran-stripped calf serum (CDSCS), and incubated for 3 days. After 3 days, fresh phenol red-free DMEM with 5% CDSCS was added to the cells along with the compounds to be tested. For controls, we used ethanol, corn oil, Vista LPA solvent (Candea Vista Co., Houston, TX), 17 β -estradiol (E₂; Sigma, St. Louis, MO), and ICI 164,384 (a gift from A. Wakeling, Imperial Chemical Industries, Macclesfield, UK). Purity of the pyrethroid compounds [sumithrin (95.4% pure), fenvalerate (95.9% pure), *d-trans* allethrin (96.5% pure), and permethrin (95.7% pure)], was taken into account when determining concentrations. Sumithrin and permethrin were suspended in LPA solvent, and fenvalerate was solubilized in corn oil. *d-trans* Allethrin was liquid at room temperature. The pyrethroid compounds were provided by McLaughlin Gormley King Co., Minneapolis, Minnesota. Cells were incubated with the various compounds for 48 hr. Total RNA was extracted using TRIzol reagent (Gibco BRL) according to the manufacturer's protocol. Total

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RNA (20 μ g) was fractionated on a 1% agarose gel containing formaldehyde and transferred to Nytran (Schleicher & Schuell, Keene, NH) by capillary action. Membranes were prehybridized with Rapid Hyb buffer (Amersham, Arlington Heights, IL) at 65°C for 2 hr. A radiolabeled probe was added to the buffer (5×10^6 cpm/ml buffer, final concentration), and hybridization was performed overnight at 65°C. Membranes were washed for 20 min with $2 \times$ standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), at room temperature followed by two washes for 15 min with $1 \times$ SSC, 0.1% SDS, at 65°C; membranes were then autoradiographed. A cloned pS2 fragment (ATCC) and a cloned β -actin fragment were used as hybridization probes for Northern analysis. Probes were labeled by random priming following the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN). Densitometric analysis was performed using a BioRad Imaging Densitometer (GS-670; Bio-Rad, Hercules, CA) and Molecular Analyst software (Bio-Rad). To account for variations in RNA loading, pS2 values were normalized to

the respective actin values. Results are expressed as the ratio of compound tested over the vehicle control (onefold) and as the mean \pm standard deviation (SD) of several independent assays. Comparisons of pS2 expression were made using the *t*-test (two-tailed, equal variance) with SAS software (SAS Institute, Cary, NC).

MCF-7 cell proliferation assay. MCF-7 cells (20,000/well) were seeded into 24-well plates and were allowed to attach for 24 hr. The medium was then replaced with phenol red-free DMEM containing CDSCS along with six concentrations of the pyrethroid compounds (0.001–100 μ M). Cell proliferation was determined on day 6 by counting the number of cells in each well using a Coulter Counter Apparatus (model Z1; Coulter Electronics, Hialeah, FL). All assay results are expressed as the mean \pm SD of three independent assays performed in duplicate. Comparisons of MCF-7 cell proliferation results as a function of concentration were made using ANOVA analyses (Bonferroni statistic for multiple comparisons) with SAS software.

Results

Based on a previous study using similar compounds on endometrial cells (17), we tested four pyrethroid compounds at a concentration of 30 μ M and the solvents used in the commercial preparations as vehicles for each compound. Two compounds, sumithrin and fenvalerate, induced pS2 expression approximately fourfold as compared to the 10-nM E_2 -positive control, which induced pS2 expression approximately fivefold. *d-trans* Allethrin and permethrin did not significantly induce pS2 expression above the negative control. The solvents, either a petroleum distillate (LPA solvent) or corn oil, used as vehicles for the various compounds were also tested and were found to have neither estrogenic nor inhibitory activity (Fig. 2).

To validate their estrogenic response, either sumithrin or fenvalerate was combined with ICI 164,384, a pure antiestrogen. Our results show that 1 μ M ICI 164,384 was sufficient to inhibit E_2 (10 nM)-induced pS2 expression (Fig. 3). Likewise, 1 μ M ICI 164,384 also inhibited 30 μ M sumithrin

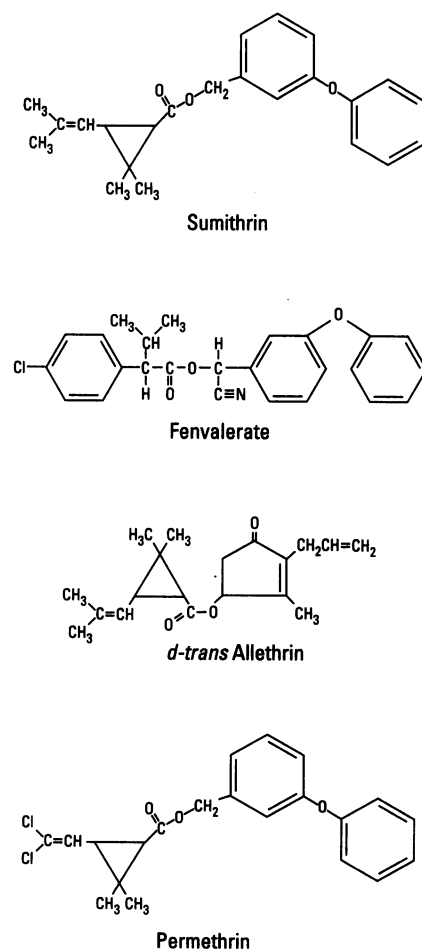


Figure 1. Chemical structures of sumithrin, fenvalerate, *d-trans* allethrin, and permethrin.

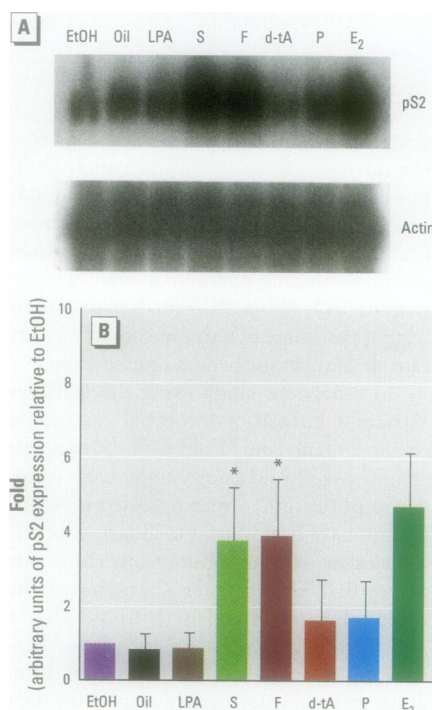


Figure 2. (A) Northern and (B) densitometric analyses of pS2 expression levels in MCF-7 cells treated with pyrethroid compounds. Sumithrin (S), fenvalerate (F), *d-trans* allethrin (d-tA) and permethrin (P) were tested at 30 μ M; EtOH, corn oil (oil), or a petroleum distillate (LPA) were used as negative controls, and 10 nM 17 β -estradiol (E_2) was used as a positive control. The results are the mean and standard deviation of five independent assays.

*Pyrethroid compounds with significantly higher levels of pS2 expression than their vehicles (S in LPA: $t = 3.27$, $p < 0.05$; F in oil: $t = 2.58$, $p < 0.05$).

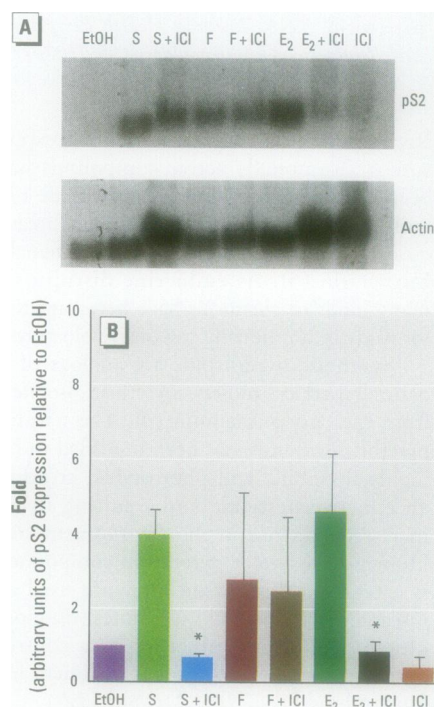


Figure 3. (A) Northern and (B) densitometric analyses of pS2 expression levels in MCF-7 cells treated with pyrethroid compounds. Sumithrin (S; 30 μ M), fenvalerate (F; 30 μ M) 17 β -estradiol (E_2 ; 10 nM) or these compounds in combination with 1 μ M ICI 164,384 were assayed, and EtOH and 1 μ M ICI 164,384 were used as negative controls. The results are the mean and standard deviation of three independent assays.

*Pyrethroid compounds + ICI 164,384 and E_2 + ICI control with significantly lower levels of pS2 expression than the compounds alone (S+ICI vs. S: $t = 9.58$, $p < 0.05$; F+ICI vs. F: $t = 1.23$, $p > 0.10$; E_2 +ICI vs. E_2 : $t = 5.77$, $p < 0.05$).

from inducing pS2 expression. In contrast, the effect of fenvalerate on pS2 expression in MCF-7 cells was not affected by the addition of ICI 164,384.

Dose-response curves were performed with each compound to determine the minimum concentration necessary to induce pS2 expression. As shown in Figure 4, concentrations of sumithrin between 1 and 750 nM were able to increase pS2 expression twofold. At concentrations between 10 and 50 μ M, sumithrin was able to significantly induce pS2 expression to the level induced by 10 nM E_2 (10-fold). Above 1 μ M, fenvalerate induced pS2 expression to levels equivalent to those induced by E_2 (Fig. 4).

Since our initial studies showed that 30 μ M *d-trans* allethrin did not stimulate pS2 expression, we examined whether this pyrethroid compound could inhibit pS2 gene expression (Fig. 5). When 5 μ M *d-trans* allethrin was combined with E_2 (0.1 nM), it inhibited E_2 induction of pS2 by 50%. In the pS2 assays, 0.1 nM E_2 was sufficient to stimulate pS2 expression to maximal levels (see Fig. 4). To further investigate *d-trans* allethrin inhibition of E_2 induction of pS2, we performed a *d-trans* allethrin dose curve in the absence or presence of 0.1 nM E_2 . At concentrations between 50 nM and 10 μ M, *d-trans* allethrin alone induced pS2 expression above basal levels (Fig. 4C). At concentrations above 50 μ M, this compound was toxic to the MCF-7 cells. Moreover, when increasing amounts of *d-trans* allethrin were combined with 0.1 nM E_2 , *d-trans* allethrin was able to significantly inhibit E_2 induction of pS2 down to basal levels.

Permethrin (5 μ M) did not induce pS2 or repress E_2 (0.1 nM) induction of pS2 in initial experiments (Fig. 5). At higher concentrations, permethrin weakly induced pS2 expression in a pattern similar to *d-trans* allethrin (Fig. 4). In combination with 0.1 nM E_2 , permethrin had little effect on pS2 expression levels stimulated by E_2 .

Another hallmark of estrogenic activity is the ability to induce MCF-7 cell proliferation. MCF-7 cells were treated with the pyrethroid compounds, and cell proliferation was determined on day 6 during the logarithmic growth phase (Fig. 6). The LPA solvent, corn oil, and ethanol were tested for their ability to affect MCF-7 cell proliferation. All three solvents were unable to stimulate cell proliferation (data not shown). Because ethanol was the primary solvent used for all the pyrethroid compounds, we used ethanol as the negative control in our assays. Both sumithrin and fenvalerate exhibited a strong dose-response trend toward inducing significant levels of cell

proliferation with increasing concentrations. At a concentration of 10 μ M, sumithrin, fenvalerate, and *d-trans* allethrin induced cell proliferation comparable to the 10 μ M E_2 ; these increases were statistically significant for all but sumithrin. At concentrations below 10 μ M, permethrin was unable to induce MCF-7 cell growth. While fenvalerate and permethrin significantly induced proliferation at 100 μ M, sumithrin and *d-trans* allethrin were toxic at this concentration.

Discussion

Our studies suggest that certain compounds of the pyrethroid family possess estrogenic properties and may function as xenoestrogens. Although estrogen is able to directly stimulate pS2 gene expression and MCF-7 cell proliferation, Kida et al. (21,22) have shown that the induction of pS2 expression is not necessarily correlated with cell proliferation. Using two different assays to test the estrogenic potential of each pyrethroid compound, we found that sumithrin was able to induce both pS2 gene expression and MCF-7 cell proliferation at nanomolar concentrations, maximally at 10 μ M. In contrast, 500 nM fenvalerate was sufficient to stimulate pS2 expression to levels similar to the 10 nM E_2 control, but 10 μ M fenvalerate was required to significantly induce cell proliferation. Furthermore, we found that *d-trans* allethrin above 1 μ M was a moderate inhibitor of pS2 expression induced by E_2 , but could significantly induce cell proliferation at 10 μ M. Permethrin only slightly affected either end point by exhibiting modest increases in pS2 expression and cell proliferation at 100 μ M. The concentrations we used are similar to the amounts necessary to observe the estrogenic properties of various chemicals including *o,p'*-DDT, pesticides, and some polychlorinated biphenyls (PCBs) in various estrogen responsive bioassays. Whereas the trends produced by each pyrethroid compound are constant, a degree of variability exists as a result of the differences in the MCF-7 cellular response from one experiment to another. Moreover, at the higher concentrations used, there was a degree of toxicity to the cells from the pyrethroid compounds that affected the outcome of the assays. This variability can be seen in both the pS2 expression and cell proliferation assays. Because of this variability, both negative and positive controls were performed for every independent assay. Within each assay, the induction of pS2 expression or cell proliferation by each pyrethroid compound remained consistent.

Our studies also revealed that induction of pS2 expression by sumithrin and possibly fenvalerate in MCF-7 cells can be blocked

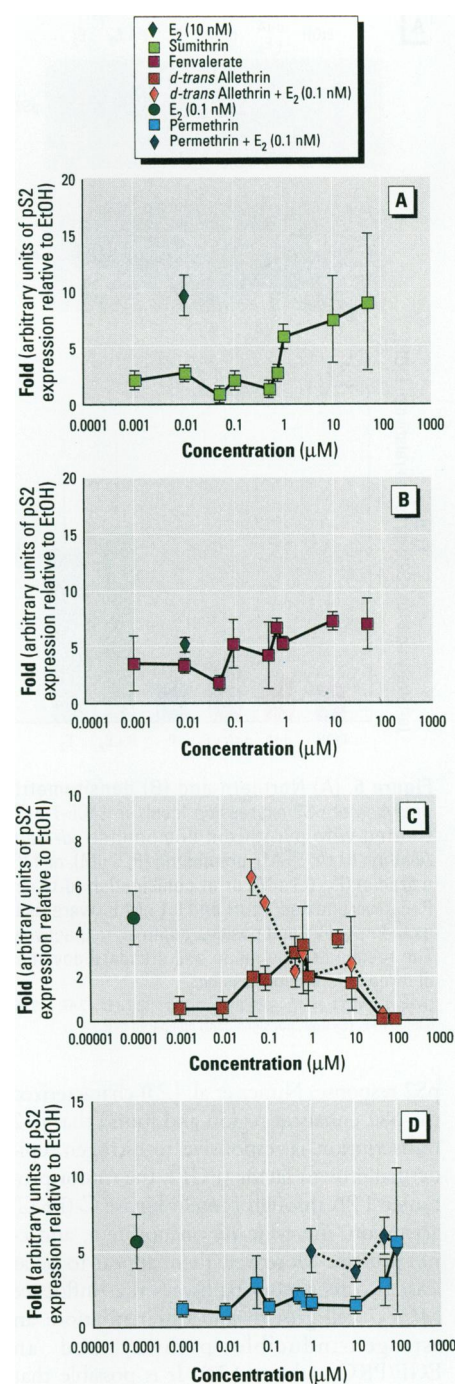


Figure 4. Dose response of pS2 expression levels in MCF-7 cells by (A) sumithrin; (B) fenvalerate; (C) *d-trans* allethrin alone or *d-trans* allethrin with 17 β -estradiol (E_2); and (D) permethrin alone or permethrin with E_2 . Each data point represents the mean and standard deviation of three independent experiments.

by ICI 164,384. This suggests that these pyrethroid compounds may act through the classical estrogen response pathway via the estrogen receptor. Moreover, fenvalerate may affect pS2 expression through an alternative signaling pathway because ICI 164,384 did not significantly inhibit fenvalerate-induced

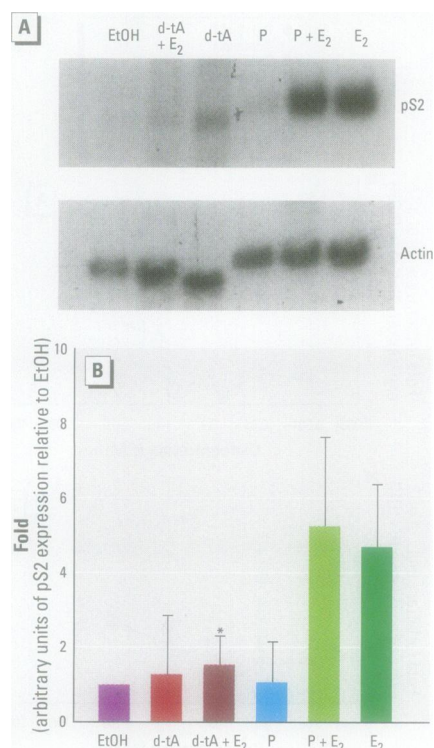


Figure 5. (A) Northern and (B) densitometric analyses of pS2 expression levels in MCF-7 cells treated with pyrethroid compounds. *d-trans* Allethrin (d-tA; 5 μ M), permethrin (P; 5 μ M), or 17 β -estradiol (E₂; 0.1 nM) or in combination (d-tA+E₂, P+E₂) were tested. EtOH and 0.1 nM E₂ were used as the negative and positive controls, respectively. The results are the mean and standard deviation of three independent assays.

*A+E₂ showed a significantly reduced expression ($t = 3.47$, $p < 0.05$), but not P+E₂ ($t = 0.40$, not significant) compared to E₂.

pS2 response. Nunez et al. (23) characterized the pS2 enhancer region and found that pS2 transcription is responsive to estrogen, epidermal growth factor (EGF), the tumor promoter TPA through protein kinase C (PKC) activation, the *c-Ha-ras* oncoprotein, and *c-jun* protein. Moreover, there appear to be at least two regulatory pathways that influence MCF-7 cell growth and differentiation: an estrogen-inducible pathway and an EGF/PKC pathway (24). It is possible that fenvalerate may be functioning via PKC to induce pS2 mRNA expression and cell proliferation. In contrast, *d-trans* allethrin at 1–10 μ M was able to antagonize estrogen induction of pS2 expression in a dose-dependent manner, possibly functioning as a competitive inhibitor of estrogen or by down regulating ER activity (25).

Our data suggest that structure–activity relationships (SARs) of these pyrethroid compounds with estrogen and estrogen metabolites may explain their various estrogenic responses in MCF-7 cells. Both DDT and pyrethroid compounds function as neurotoxins, and structural comparison between

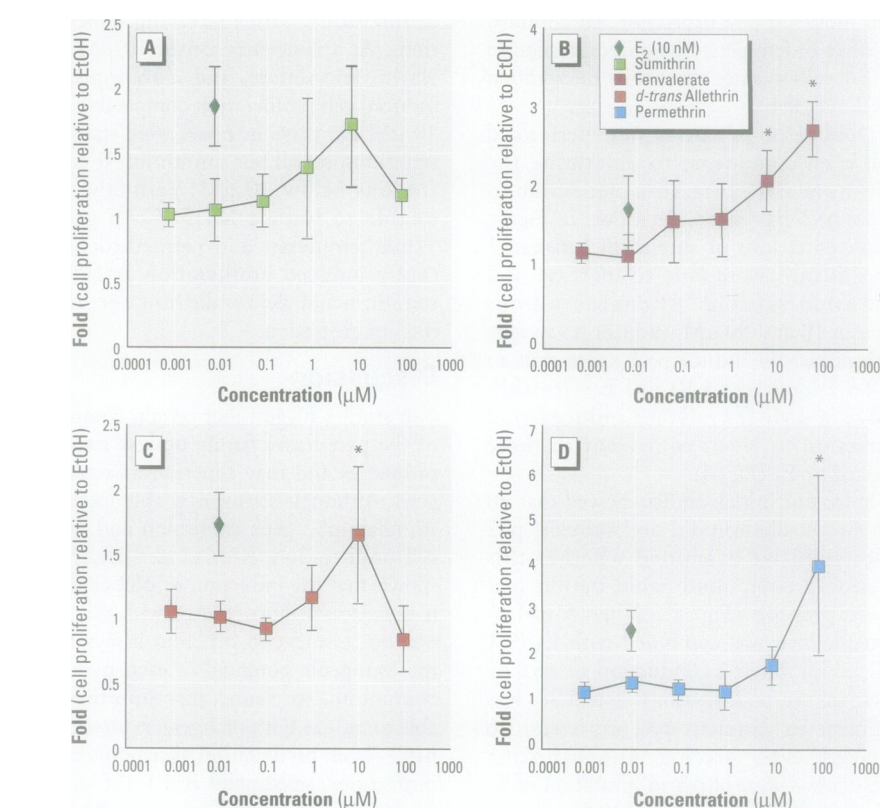


Figure 6. Dose response of MCF-7 cell proliferation by (A) sumithrin; (B) fenvalerate; (C) *d-trans* allethrin; or (D) permethrin. 17 β -Estradiol (E₂; 10 nM) was used as the positive control. Values shown are means \pm standard deviations.

*Significant increase in cell proliferation compared with other concentrations (Bonferroni statistic, $p < 0.05$).

the two types of chemical compounds has shown that some pyrethroids and DDT share similar structural properties and may act on similar or the same neuroreceptors (6). Additionally, a study on SARs of the PCB family of chemical compounds showed that the ability of PCBs to activate PKC is dependent on the spatial orientation of the biphenyl moieties and on the degree and pattern of chlorination (26). Perez et al. (27) recently proposed that both the length and the nature of the substituent groups at the bridging carbon of bisphenol A-related diphenylalkanes affected the degree of estrogenicity of these chemical compounds. Therefore, we propose that certain pyrethroid compounds that are partially composed of biphenyl ether moieties may signal through PKC to affect pS2 mRNA expression and cell proliferation. Equally important, some pyrethroid compounds may possess critical structural properties similar to estrogen metabolites. Estrogen and its metabolites are thought to affect DNA binding, induce DNA damage and DNA repair, and alter the amounts of enzymes necessary to clear the metabolites (28). Pyrethroids have structural components similar to estrogen metabolites and polyphenols that may explain their estrogenic response. Whether this is the case remains to be determined.

Consistent with previous reports showing that certain pyrethroid compounds are able to affect the endocrine system, we confirmed that some pyrethroids (sumithrin, fenvalerate, and *d-trans* allethrin) are able to disrupt estrogen function, whereas another pyrethroid, permethrin, does not effect estrogen regulation. Further *in vitro* and *in vivo* research is required to elucidate the mechanisms by which these novel xenoestrogens function. Nevertheless, pyrethroids are widely used, are prevalent in the environment, and can alter estrogen homeostasis. Therefore, their effects on the endocrine system in both humans and wildlife is of concern.

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